Inflammation-related induction of absent in melanoma 2 (*AIM2*) in vascular cells and atherosclerotic lesions suggests a role in vascular pathogenesis

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Background: **Absent in melanoma (AIM2) was recently identified to act as a cytosolic DNA sensor in innate immunity. Considering the role of chronic inflammation in atherosclerosis, we hypothesized that AIM2 may act as a damage signal that is activated in response to cellular stress likewise in vascular cells of larger arteries. We thus addressed AIM2 expression in healthy arterial wall and in different vascular lesions. In addition, AIM2 expression was characterized in cultured human aortic endothelial cells (HAoECs), smooth muscle cells (HAoSMCs), and T/G-HA-vascular smooth muscle cells (VSMCs) in response to different stimuli.**

Methods: **Carotid and aortic lesions from patients who underwent surgery and normal arterial specimens were analyzed by immunohistochemistry for AIM2 expression. Cultured HAoECs, HAoSMCs, and T/G-HA-VSMCs were stimulated in vitro with proinflammatory cytokines (tumor necrosis factor-α, interferon-γ) or poly(dA:dT) and analyzed for AIM2 transcript and protein expression.**

Results: **AIM2 was detected in ECs of the intima and vasa vasorum of normal carotid artery and aorta. Moreover, AIM2 was moderately expressed in VSMCs of normal media and intima layers, as well as in VSMCs of atherosclerotic lesions. Increased AIM2 expression was detected around the necrotic core of atherosclerotic carotid lesions and in the vasa vasorum neovasculature of aortic aneurysms. Subsequent in vitro analysis identified an endogenous *AIM2* expression in cultured HAoECs, HAoSMCs, and T/G-HA-VSMCs that was markedly increased upon treatment of the cells with tumor necrosis factor-α, interferon-γ, or cytosolic DNA.**

Conclusions: **ECs and VSMC are able to respond to inflammatory signals by upregulation of AIM2 expression, indicating a role of AIM2 in vascular pathogenesis. (J Vasc Surg 2014;59:794-803.)**

Clinical Relevance: **This study revealed a prominent role of the interferon-γ–inducible double-stranded DNA sensor absent in melanoma (AIM2) in vascular inflammation. Immunohistochemical analysis suggested increased AIM2 expression in smooth muscle cells of atherosclerotic carotid lesions and the vasa vasorum of abdominal aortic aneurysms. In vitro stimulation of aortic endothelial cells and smooth muscle cells by inflammatory cytokines and double-stranded DNA dramatically increased AIM2 expression, which indicates a role of AIM2 in response to inflammatory signals in these cells. These findings provide new insight on the molecular mechanisms of vascular inflammation beyond the role of AIM2 in macrophages and thus widen the range of putative prognostic and therapeutic targets in vascular pathogenesis.**

According to current concepts, atherosclerosis is considered a complex biologic reaction to vascular damage that is accompanied by inflammatory mechanisms.[1] Growing evidence suggests a role of the innate immune system in degenerative processes within the arterial wall.[2] Macrophages, infiltrating early atherosclerotic lesions, were shown to release interleukin (IL)-1β upon activation of caspase-1–containing protein complexes, called inflammasomes.[3] Inflammasome activation was shown to be involved in neointima formation after vascular injury.[4,5] Furthermore, cholesterol crystal deposition appears to act as an endogenous danger signal. In infiltrating macrophages, the crystals activate a nucleotide-binding oligomerization domain (NOD)-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome, which is required for atherogenesis.[6,7] Besides infiltrating inflammatory cells, endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) appear to respond with molecular defense mechanisms to mechanical and physiologic stress. In particular, inducible expression of Toll-like receptors and endogenous pathogen-associated pattern recognition receptors has been detected in vascular endothelium in response to injury, resulting in activation of

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caspase-1 and release of the proinflammatory cytokines IL-1β and IL-18.[8]

Recently, absent in melanoma 2 (AIM2) was identified as a novel inflammasome-activating protein that acts as a cytosolic DNA sensor in macrophages.[9-12] AIM2 belongs to the HIN200 family of hematopoietic, interferon (IFN)-inducible, nuclear proteins and appears to be essential for host defense against DNA viruses and certain cytosolic bacteria.[13-16] In addition, we and others demonstrated a role of AIM2 in tumor pathology.[17-20] AIM2 is frequently affected by genetic and epigenetic alterations in different human tumor entities.[18,21,22] Moreover, overexpression or induction of AIM2 by IFN-γ in tumor cells resulted in reduced cell proliferation, increased cell migration,[19] and induction of IFN-γ–stimulated target genes.[20] The role of AIM2 in nonmyeloid vascular cells, however, has not yet been investigated.

Given the above-described role of inflammasomes in vascular dysfunction and our previous findings in tumor cells, we assumed that AIM2 might likewise act as a potential damage receptor for vascular injury. We thus addressed AIM2 expression in healthy arterial wall and in different arterial lesions with respect to cellular origin, expression intensity, and intraplaque localization. Moreover, AIM2 expression was characterized in cultured human aortic ECs and VSMCs in response to proinflammatory cytokines and cytoplasmic DNA.

METHODS

All tissues used in this study were collected and processed according to ethical guidelines. The University of Heidelberg Medical Ethics Committee approved this study. All patients gave their written informed consent.

Tissues. Four aortic artery and 20 carotid artery lesions from patients undergoing surgery were randomly selected from the vascular tissue bank of the Department of Vascular Surgery, Heidelberg, Germany. Normal vascular tissue derived from cadaveric donors was obtained from the tissue bank of the National Centre for Tumor Disease (NCT-Gewebebank Heidelberg). Basic demographic and clinical data of patients are listed in the Supplementary Table (online only).

Tissue processing. Carotid artery plaques from patients undergoing carotid endarterectomy were obtained by dissection of the internal carotid artery from the bifurcation and harvesting by the vascular surgeon (routine eversion technique). Indications for carotid endarterectomy were high-grade internal carotid artery stenosis (as determined by ultrasound imaging), transient ischemic attack, amaurosis fugax, or ischemic stroke. Aortic tissue samples were harvested by the vascular surgeon from patients undergoing open infrarenal aortic aneurysm repair after proximal aortic cross-clamping. Indication for infrarenal aortic aneurysm repair was a maximum aneurysm diameter of >50 mm and exclusion from endovascular repair.

Four aortic samples showing signs of atherosclerosis, such as cholesterol inclusion and calcification, were chosen for analysis. Samples were immediately rinsed with saline to remove surface blood and divided into two tissue blocks, one for formalin fixation and a second for shock freezing in liquid nitrogen and cryoconservation at −80°C.

Cell culture. Primary human aortic ECs (HAoECs) and smooth muscle cells (HAoSMCs) were purchased from PromoCell (Heidelberg, Germany). Cells were grown in Endothelial Cell Growth Medium MV, supplemented with Endothelial Cell Supplement Mix or in Smooth Muscle Cell Growth Medium (PromoCell), respectively. The HAo-VSMC cell line T/G HA-VSMC (LGC Standards, Wesel Germany) was cultivated in human VSMC medium 231 supplemented with SMGS (Life Technologies, Darmstadt, Germany). Subcultures were obtained every 2 or 3 days by using DetachKit-125 (PromoCell) for five passages (HAoECs and HAoSMCs) or longer (T/G HA-VCMCs). For stimulation, cells were seeded in 6-well plates at a density of 10^6 cells/well and 100 U/mL recombinant human IFN-γ (R&D Systems, Wiesbaden-Nordenstadt, Germany), 25 ng/mL recombinant human TNF-α (R&D Systems) or 1 μg/mL poly(dA:dT) (poly(dA:dT)/LyoVec, InVivo) was added to the media for 6 to 96 hours, as indicated in Figs 1 and 2.

The timing and dosage of stimulation with cytokines that were chosen for analysis of AIM2 expression had been previously investigated in tumor cells.[20] Treatment of the cells with the indicated dosages progressively reduced proliferation and resulted in cellular senescence after 96 hours (Lee et al[20] and unpublished data). The amount of poly(dA:dT) was optimized in preliminary experiments (Supplementary Fig 1, online only). Stimulation was repeated every 48 hours by replacement of the media and addition of fresh cytokines. Independent of the stimulation period, all cells were grown for 96 hours on 6-well plates before harvesting for RNA extraction or lysis in radioimmunoprecipitation assay buffer (see below).

The monocyte cell line THP-1 was grown in Roswell Park Memorial Institute-1640 cell culture medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics. To induce differentiation, cells were treated with 300 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, Taufkirchen, Germany) for 3 hours, washed in phosphate-buffered saline, grown for 3 days, and transfected overnight with 1 μg poly(dA:dT).

Immunohistochemistry. Tissues were formalin-fixed and embedded in paraffin in transversal orientation according to standard procedures for conventional histologic analysis. For immunohistochemical detection of protein expression, serial 4-μm sections were prepared from each paraffin-embedded tissue specimen. After paraffin was removed and samples were rehydrated, they were pretreated according to individually optimized protocols (detailed protocols are available on request). The primary antibodies used for detection were polyclonal rabbit anti-AIM2 (dilution 1:150; Sigma, Taufkirchen, Germany), monoclonal mouse anti-SMA (clone 1A4; dilution 1:1000; DAKO, Glostrup, Denmark), monoclonal mouse anti-CD68 (clone PG-M1; dilution 1:1000, DAKO), and monoclonal mouse anti-CD31 (clone 89C2;

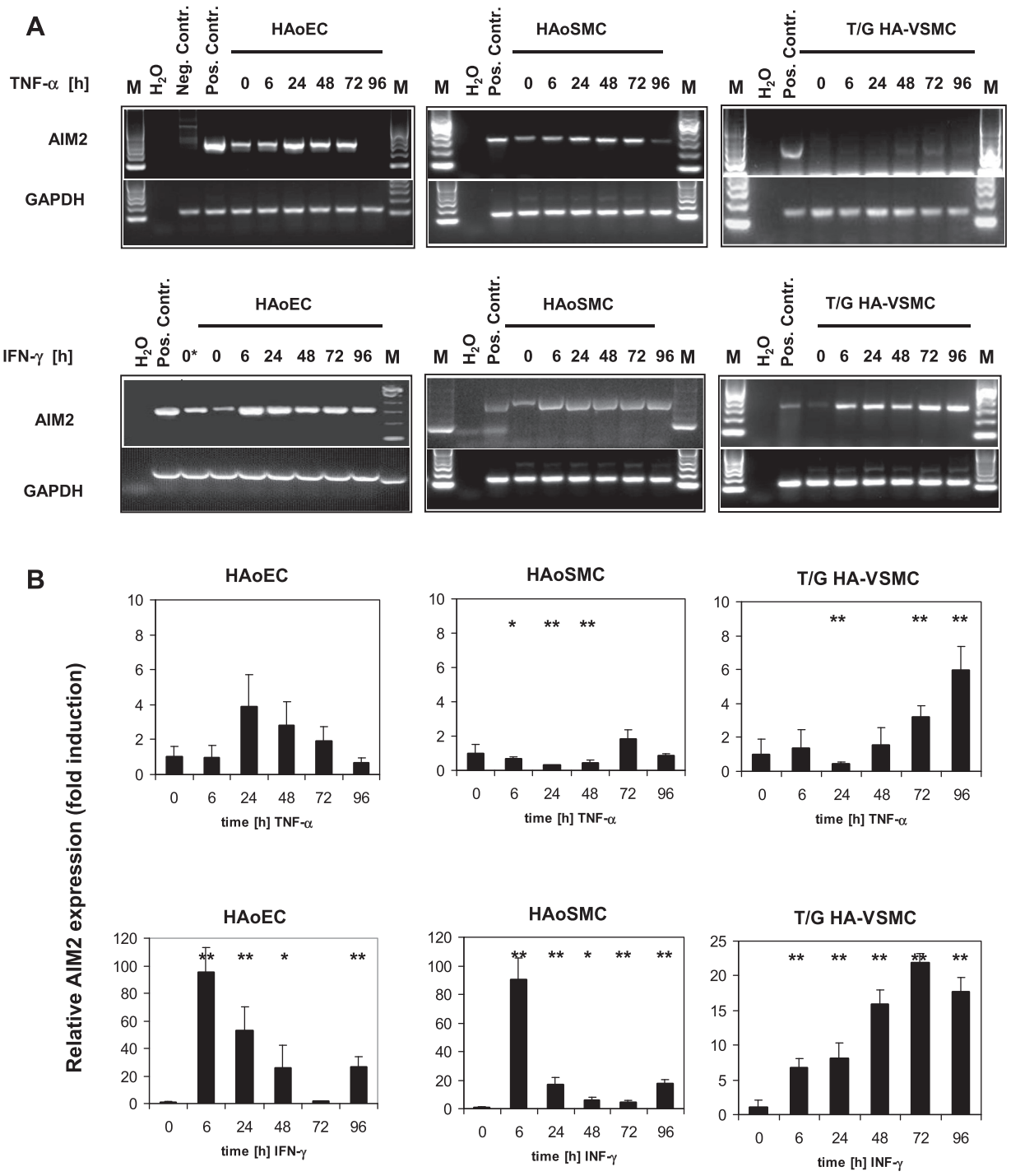

Fig 1. *Absent in melanoma 2* (AIM2) transcript expression in human aortic endothelial and smooth muscle cells in response to inflammatory cytokines illustrating the time-dependent induction of *AIM2* in each of the cell types. *Glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) expression of corresponding samples is shown for control to demonstrate that equal amounts of total complementary DNA were analyzed, independent of cell viability. **A,** End-point reverse-transcription polymerase chain reaction of tumor necrosis factor (*TNF*)-α–stimulated (*upper panel*) and interferon (*IFN*)-γ–stimulated (*lower panel*) human aortic endothelial cells (*HAoEC*), smooth muscle cells (*HAoSMC*), and in the aortic vascular smooth muscle cell line (*T/G-HA-VSMC*). **B,** Quantitative real-time reverse transcription polymerase chain reaction of the same cells as in **A**. Fold-*AIM2* expression is shown relative to expression of 18s RNA in corresponding samples. The *bars* and *error bars* represent the mean and standard deviation, respectively, of triplicate analysis. $*P < .05$; $**P < .01$ (paired *t*-test) 0*: cells were grown without media replacement for 96 hours.

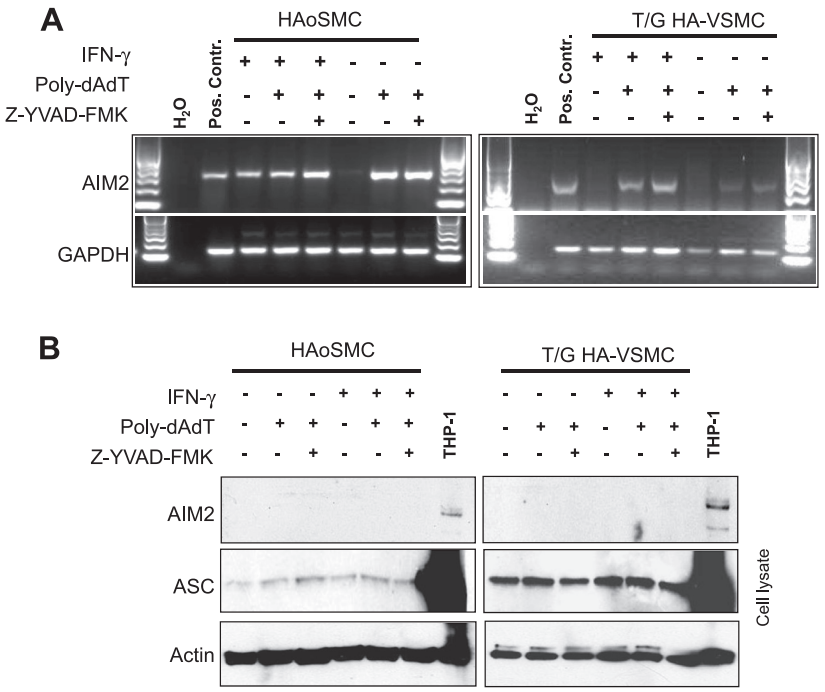

Fig 2. Expression of **(A)** absent in melanoma 2 (*AIM2*) transcript and **(B)** protein in human aortic smooth muscle cells (*HAoSMC*) upon stimulation with foreign double-stranded DNA (*poly(dA:dT)*) with or without previous stimulation with interferon (*IFN*)-γ. **A,** Little *AIM2* expression is detected in unstimulated cells, which is markedly upregulated upon transfection of the cells with poly(dA:dT) and pretreatment of the cells with IFN-γ. **B,** The inflammasome component ASC is expressed in HAoSMC and in the aortic vascular smooth muscle cell line (*T/G-HA-VSMC*) at levels that are not affected by cytoplasmic double-stranded DNA or IFN-γ. AIM2 protein expression is below detection level in HAoSMC and T/G-HA-VSMC.

dilution 1:100; Cell Signaling Technologies, New England Biolabs, Frankfurt, Germany).

RNA extraction, complementary DNA synthesis, and polymerase chain reaction analysis. RNA was extracted from indicated cell lines using the RNeasy Kit (Qiagen, Hilden, Germany). For complementary (c)DNA synthesis, 1 μg total RNA from each sample was reverse-transcribed using oligo-dT primers and SuperScript II reverse transcriptase (Invitrogen Life Technologies, Karlsruhe, Germany), following the manufacturer's instructions. End-point polymerase chain reaction (PCR) was performed as previously described[18] by using the following primers:

AIM2: 5′-AGCTGACATCTGGAGTTCATAGC-3′ and 5′-CTGCTTAGACCAGTTGGCTTG-3′;
GAPDH: 5′-GGCTGCTTTTAACTCTGGTA-3′ and 5′-CTTGACGGTGCCATGGAATT-3′.

For real-time PCR, PowerSYBR Green Master Mix (Applied Biosystems, Darmstadt, Germany) was added to appropriate cDNA samples and primers:

AIM2: 5′-CATCTGCAGCCATCAGAA-3′ and 5′-CGCTTCTGAAACCCTTCTCT-3′;
18s RNA: 5′-AAACGGCTACCACATCCAAG-3′ and 5′-CCTCCAATGGATCCTCGTTA-3′.

Samples were loaded onto 96-well PCR plates and analyzed in an ABI Prism 7300 thermocycler (Applied Biosystems), as described.[20] Quantitative analysis of gene expression was performed relative to expression of 18s RNA in corresponding samples by using individual standard amplification curves of each transcript.

Immunoblotting. Cells were harvested on ice in radioimmunoprecipitation assay cell lysis buffer (50 mM TRIS-HCl [pH 7.5], 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.5% NP-40) supplemented with protease inhibitor (Mix G, Serva, Heidelberg, Germany). Equal amounts of protein (20-30 μg per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted on nitrocellulose, and visualized by chemoluminescence, as described.[20] The following antibodies and dilutions were applied for detection: rabbit polyclonal anti-AIM2 (ab93015, dilution 1:500; Abcam, Cambridge, United Kingdom), antiactin (clone C4, dilution 1:5000; MP Biomedicals, Aurora Ohio), and rabbit polyclonal anti-ASC (dilution 1:1000; Enzo Life Sciences, Lörrach, Germany).

RESULTS

AIM2 is differentially expressed in healthy and diseased arterial wall. We initially investigated the AIM2 expression pattern in normal vs diseased human arterial

wall of different pathologic entities (atherosclerotic carotid plaques and arterial tissue from abdominal aortic aneurysms) to receive an impression about its localization and cell affiliation. By immunohistochemical analysis using thoroughly characterized antibodies (Supplementary Fig 2, online only), we found basic AIM2 expression in intact, nonatherosclerotic intima and media from the internal carotid artery and the aorta (representative examples in Fig 3, *A* and *B*). In addition, AIM2 expression was detected in CD31-positive, as well as in smooth muscle actin (SMA)-positive cells of normal vasa vasorum, indicating that EC and VSMC can both endogenously express AIM2 in situ (Fig 3, *A* and *B*).

In contrast, the AIM2 expression pattern was more complex in vascular lesions. Three of four abdominal aortic aneurysms displayed increased AIM2 expression in SMA-positive cells of intima/media and vasa vasorum/neovascular cells, suggesting that it is upregulated in VSMC in these lesions compared with intact aortic wall (Fig 4, *A*, *lesions 1-3*). Surprisingly, AIM2 expression was absent in the SMA-positive intima/media of one aortic lesion (Fig 4, *A*, *aortic lesion 4*). In 16 of 20 atherosclerotic carotid lesions, a particularly high AIM2 expression was found around the necrotic core of the plaque (representative examples in Fig 4, *B*), where it overlapped with expression of SMA and CD68. The latter identifies macrophages infiltrating the lesion, which suggests that at least some of the AIM2-positive cells represent inflammatory cells.

***AIM2* expression in cultured HAoECs and HAoSMCs is induced by TNF-α and IFN-γ.** To further elucidate the identity of AIM2 expressing cells, we analyzed cultured primary HAoECs, primary HAoSMCs, and the T/G HA-VSMC cell line in vitro and investigated their capacity to respond to the proinflammatory cytokines TNF-α and IFN-γ. The rationale behind this stimulation was to test our hypothesis that cytokines released from inflammatory cells activate AIM2 or even an inflammasome in macrovascular nonmyeloid cells of larger arteries, thus provoking an intracellular damage response. Aberrant production of TNF-α and IFN-γ by activated macrophages and T cells, respectively, has been associated with several inflammatory diseases, including atherosclerosis.[23,24]

We here detected basic *AIM2* transcript expression in unstimulated primary aortic cells (HAoECs and HAoSMCs), whereas it was very low to absent in the T/G HA-VSMC cell line (Fig 1, *A*). Stimulation of the cells with TNF-α or IFN-γ resulted in a time-dependent upregulation of *AIM2* expression in each of the cell types (Fig 1, *A*), although induction by TNF-α was only weak in T/G HA-VSMC cultures (Fig 1, *A*, *upper right panel*). Quantitative analysis by real-time PCR revealed a maximum of up to 100-fold induction of *AIM2* transcript expression in HAoEC and HAoSMC cultures and 22-fold in T/G-HA-VSMC cultures in response to IFN-γ (Fig 1, *B*, *lower panel*), whereas induction by TNF-α was less intense (Fig 1, *B*, *upper panel*).

To determine AIM2 protein expression, we next performed Western blot analysis of untreated and cytokine-stimulated cultured aortic cells. The 39-kD AIM2 protein monomer was clearly upregulated in HAoEC cultures by TNF-α and IFN-γ compared with untreated HAoEC cultures (Fig 5, *A*). The maximum expression level was observed after 6 and 24 hours of treatment, respectively, and declined to the initial basic level during longer treatment (Fig 5, *A*), despite continuous replacement of media and cytokines every 48 hours. This suggests that AIM2 induction is an early, transient event in HAoECs. In contrast to HAoECs, AIM2 protein expression was hardly detectable in TNF-α and IFN-γ–stimulated primary HAoSMCs and remained below detection level in T/G-HA-VSMCs (Fig 5, *B* and *C*).

Cytosolic double-stranded DNA induces *AIM2* transcript expression in cultured HAoSMCs. In addition to its role in macrophages, AIM2 was recently shown to be induced by cytosolic double-stranded (ds)DNA likewise in nonmyeloid cells, such as keratinocytes.[25,26] Upon induction, AIM2 here triggered inflammasome activation and caspase-1–dependent release of activated IL-1β. We therefore investigated whether AIM2 inflammasomes may likewise be activated by dsDNA in VSMCs. Transfection of the cells with poly(dA:dT) clearly induced *AIM2* transcript expression in HAoSMC and T/G-HA-VSMC cultures (Fig 2, *A*). Moreover, pretreatment of the cells with 100 U/mL IFN-γ for 6 hours resulted in an additional upregulation of poly(dA:dT)-induced *AIM2* transcript expression in HAoSMC and T/G HA-VSMC cultures (Fig 2, *A*).

To further determine activation of an AIM2 inflammasome in SMC cultures, whole cell lysates treated as described with IFN-γ or poly(dA:dT), or both, were subjected to Western blot analysis for detection of several inflammasome components. Despite clear detection of the AIM2 protein in SMC in situ (Fig 3, *A*), it remained below detection level in cultured SMC, even upon stimulation (Fig 2, *B*). The adaptive protein apoptosis-associated speck-like protein containing CARD (ASC), which is required in macrophages for activation of the AIM2 inflammasome,[9] was detected in HAoSMC and T/G-HA-VSMC cultures at levels that were independent from stimulation with IFN-γ or poly(dA:dT), or both (Fig 2, *B*). However, in contrast to the findings in macrophages and keratinocytes, activation of caspase-1 and IL-1β could not be demonstrated in VSMCs, neither in response to IFN-γ or TNF-α nor in response to poly(dA:dT) (data not shown). Thus, VSMCs respond to foreign cytoplasmic DNA with upregulation of *AIM2* expression potentially without triggering caspase-1 activation.

DISCUSSION

This study characterized AIM2 expression in human nonmyeloid vascular cells and lesions of different entities and provides evidence for its role in vascular inflammation and atherogenesis. The AIM2 protein was immunohistochemically detected in human vascular cells of healthy

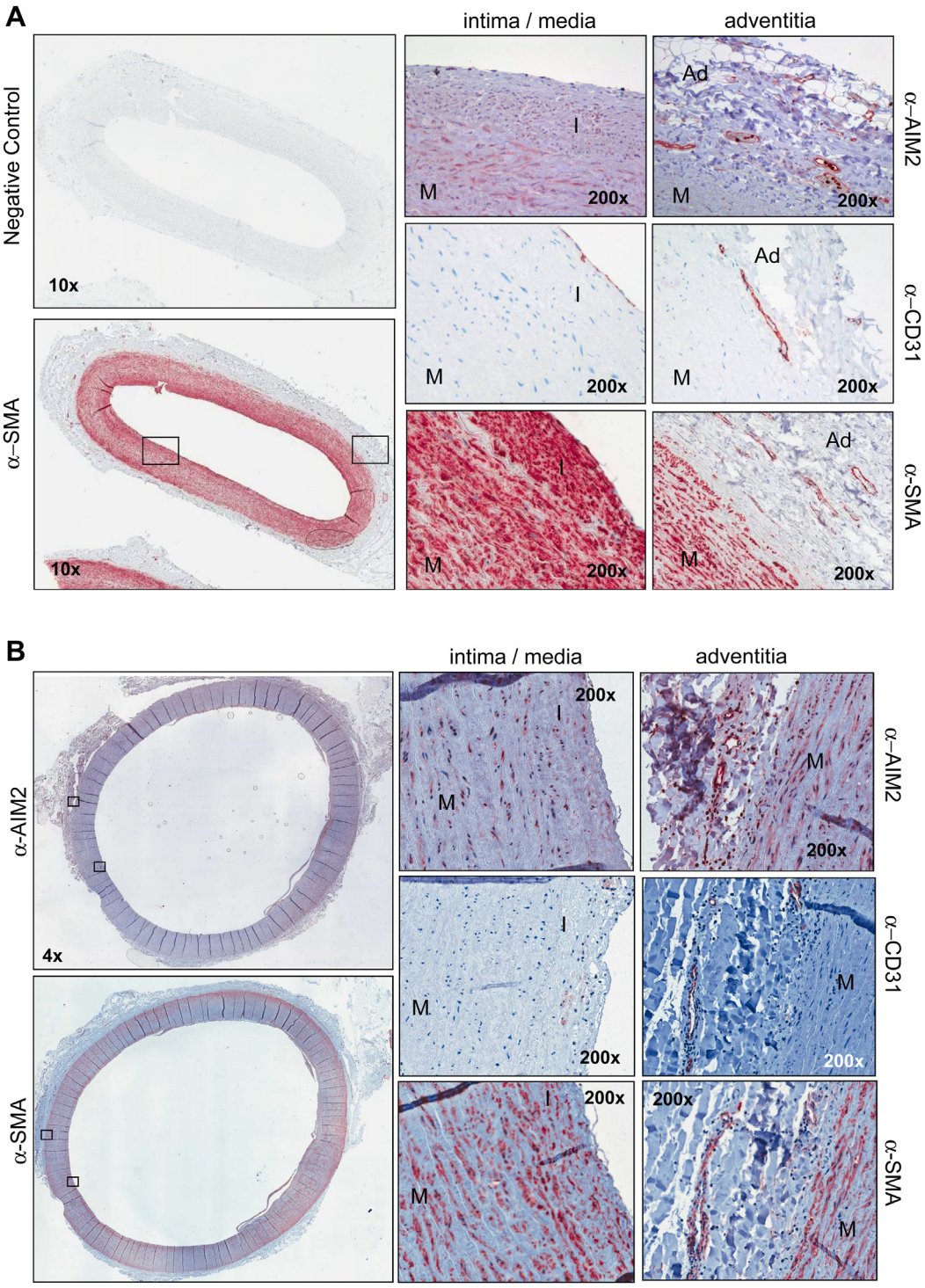

Fig 3. Absent in melanoma 2 (*AIM2*) expression pattern is shown in human arterial wall. Formalin-fixed paraffin-embedded sections were immunohistochemically stained for AIM2, CD31, and smooth muscle actin (*SMA*). **A,** Normal, nonatherosclerotic carotid artery. **B,** Normal, nonatherosclerotic aorta. *Ad,* Adventitia; *EC,* endothelial cells; *I,* intima; *M,* media. Original magnification, ×4, ×10, or ×200, respectively, as indicated. Examples from 10 normal arterial samples are shown, with *red* indicating positive immunostaining and *blue* indicating nuclei and connective tissue.

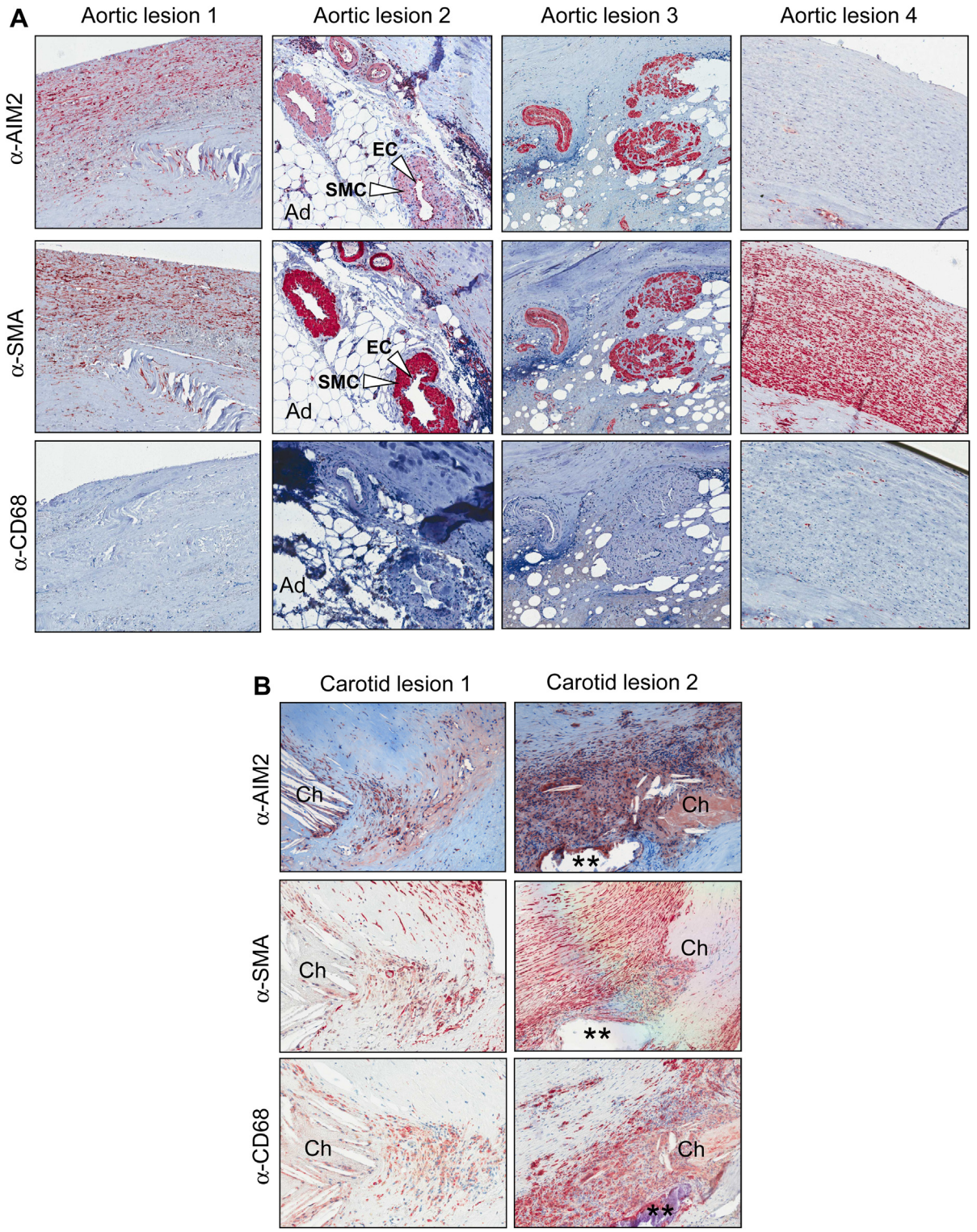

Fig 4. Absent in melanoma 2 (*AIM2*) expression pattern is shown in lesions derived from **(A)** abdominal aortic aneurism (AAA) and **(B)** atherosclerotic carotid artery. **A,** AIM2 expression is increased in smooth muscle actin (*SMA*)-positive cells of the intima/media (*lesion 1*) and of the vasa vasorum (*lesions 2* and *3*; adventitia) of AAA, whereas it is absent in the SMA-positive intima/media of *lesion 4*. **B**, Atherosclerotic carotid lesions show that AIM2 expression accumulates in cells surrounding necrotic and calcified (**) areas or cholesterol (*Ch*) within the intima/media. Examples of four AAA samples and 20 atherosclerotic carotid samples are shown, with *red* indicating positive immunostaining and *blue* indicating nuclei and connective tissue. Original magnification, ×100.

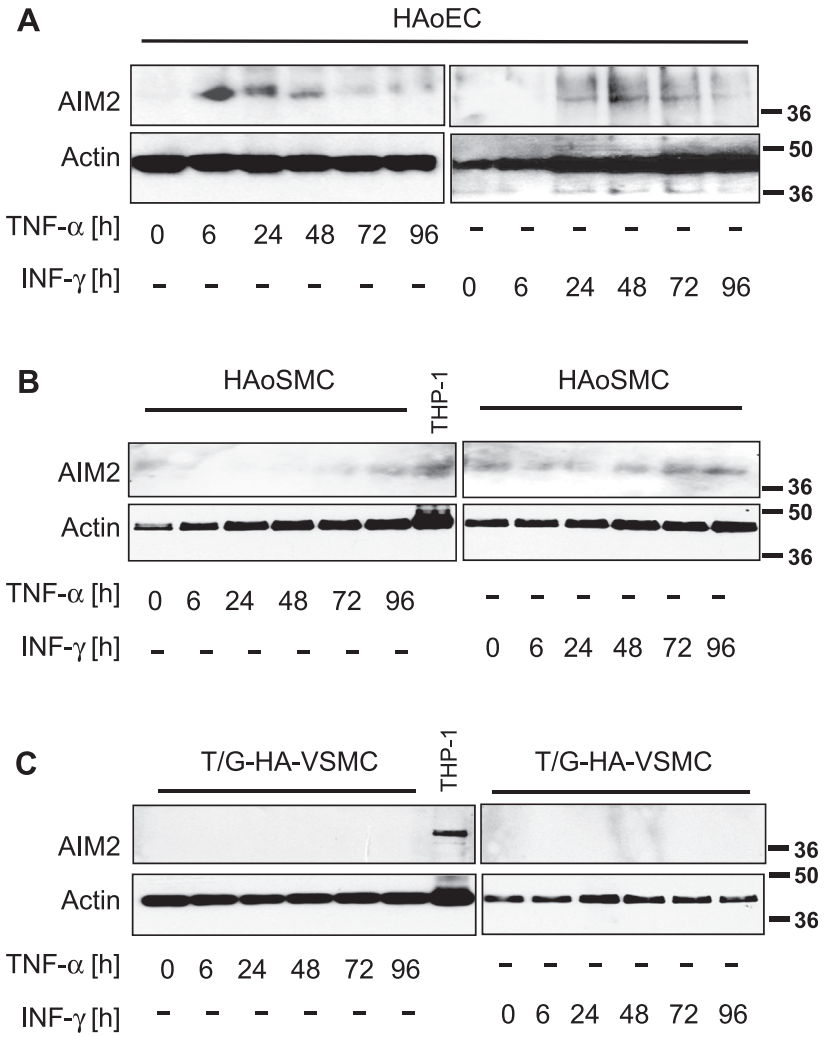

Fig 5. Western blot analysis for absent in melanoma 2 (*AIM2*) protein expression in **(A)** primary human aortic endothelial cells (*HAoEC*), **(B)** primary human aortic smooth muscle cells (*HAoSMC*), and **(C)** the aortic vascular smooth muscle cell line (*T/G-HA-VSMC*). Cells were analyzed in response to the inflammatory cytokines tumor necrosis factor (*TNF*)-α and interferon (*IFN*)-γ. Lysates were derived from cells stimulated in parallel to the cells used in Fig 1. Differentiated THP-1 cells were transfected with poly(dA:dT), as described in the Methods section, and used as a positive control. Protein loading was assessed by actin expression.

and pathologic macrovascular tissues. Moreover, the proinflammatory cytokines TNF-α and IFN-γ as well as dsDNA were identified as triggers to stimulate *AIM2* expression in aortic ECs and VSMCs, respectively. This is the first report demonstrating that ECs and VSMCs can respond to these proinflammatory stimuli by elevating AIM2 expression. The precise role of AIM2 in vascular cells, however, is not yet clear.

Similar to other nonmyeloid cells, such as keratinocytes[25,26] and certain tumor cells,[27] aortic ECs and VSMCs might express AIM2 as a component of inflammasomes. Inflammasomes are multiprotein complexes that can proteolytically activate caspase-1 and caspase-5, which results in release of IL-1β and a subsequent form of cell death, called pyroptosis. Their activity is regulated by expression of several proteins, including NLRP3, NLRC4, NLRP6, and AIM2, which act as sensors for microbial infection or cellular damage.[3] Induction of an AIM2 inflammasome resulting in caspase-1–dependent inflammatory response and release of IL-1β was originally shown to be important for pathogen defense by macrophages.[28,29] Moreover, NLRP3 inflammasomes have been demonstrated to be activated by endogenous cholesterol crystals in plaque infiltrating macrophages of atherosclerotic lesions.[6,7] Our data add to these findings by identifying AIM2 induction in aortic ECs and VSMCs in response to proinflammatory stimuli, which suggests its potential participation in inflammasome activation in these cell types.

ECs have been previously analyzed for activation of inflammasome components upon stimulation with TNF-α.[8]

The authors found slightly increased transcript expression of *NOD-like receptor family, pryrin domain-containing 1* (*NLRP1*) and *IL-1β* in human umbilical vein endothelial cells (HUVECs) treated with TNF-α for 6 hours. Furthermore, induction of several Toll-like receptor genes (*TLR1, TLR3, TLR6,* and *TLR7*) was detected, whereas *NLRP3, Caspase-1,* or *Caspase-5,* the characteristic inflammasome components of macrophages infiltrating atherosclerotic lesions, were not induced.[8] On the basis of their data, the authors suggested that in principle, upregulation of certain inflammasome-related gene expression is modulated by inflammatory signals in ECs; however, they state that future work on that is needed in additional vascular cell types and in response to further stimuli.[8] Our findings support this idea by demonstrating induction of AIM2 in TNF-α– and IFN-γ–stimulated HAoECs. Moreover, we recently identified transcript expression of additional inflammasome components in HAoECs, including *ASC, Pro-IL-1β, CASP1,* and *CASP5* (data will be published elsewhere). However, activation of these components on the protein level and their functional role in association with AIM2 remain to be investigated. We thus cannot yet conclude from our data whether an inflammasome is activated in aortic ECs stimulated by TNF-α or IFN-γ.

Alternatively, AIM2 may be involved in activation of other intracellular inflammatory pathways in this cell type. Interestingly, we had previously demonstrated upregulation of *TLR3* and several *IFN*-stimulated genes (*ISGs*) in AIM2 transfected cancer cells.[20] Our ongoing investigations will illustrate whether *ISGs* or other inflammatory response genes are likewise induced in aortic ECs upon induction of AIM2.

Finally, IFN-γ stimulation of ECs is known to enhance EC-leukocyte adherence and class 2 antigen presentation by ECs (ie, in response to infection of the endothelium by pathogens).[30] Consequently, the IFN-γ–stimulated induction of AIM2 in aortic ECs identified here may alternatively mediate this effect. Our recent findings demonstrating that AIM2 overexpression results in upregulation of human leukocyte antigen class II and of several genes involved in intercellular adhesion[20] are in line with this hypothesis. However, whether AIM2 overexpression likewise mediates induction of these genes in aortic ECs remains to be determined.

Numerous reports have described the response of VSMCs to the proinflammatory cytokines TNF-α and IFN-γ, which involves induction of gene expression and de novo protein synthesis that induces new effector functions.[31] We here observed a slightly different time course of *AIM2* induction between two aortic SMC cultures, HAoSMCs and T/G-HA-VSMCs. The reason for this discrepancy might be the origin of the two cell types. HAoSMCs are primary aortic cells derived from an 84-year-old donor (PromoCell, certificate of analysis). They undergo 10 to 15 doublings and can be passaged five to six times in culture before they stop proliferation and go into a senescent status. In contrast, T/G-HA-VSMC is a normal human aortic cell line from an 11-month-old donor, which may undergo 35 doublings before senescence (LGC standards, product description). The response to cytokines and upregulation of AIM2 is possibly differentially regulated according to cell age.

Little is known about inflammasome or IL-1β activation, or both, in VSMCs. Induction of the *NLRP3* and *IL-1β* genes was recently reported to occur in TNF-α–stimulated aortic VSMCs, indicating a role of the NLRP3 inflammasome in these cells.[32] In addition, increased transcript expression of caspase-1 (also known as interleukin-1β–converting enzyme), was reported to induce cell death of VSMCs.[33] Our data, demonstrating constitutive expression of ASC, in combination with dsDNA-/IFN-γ–stimulated induction of *AIM2* expression in VSMCs are in line with the hypothesis of an AIM2 inflammasome in VSMCs. However, as with aortic ECs, we cannot conclude from our data whether an inflammasome is activated in TNF-α– or IFN-γ–stimulated aortic VSMCs. Although AIM2 protein was detected in healthy media of carotid and aortic arteries and vasa vasorum, it remained below the detection level in cultured dsDNA-/IFN-γ–stimulated VSMC, despite a clear induction of *AIM2* transcript expression. Moreover, neither a proteolytic activation of caspase-1 nor of IL-1β was detectable in dsDNA-/IFN-γ–stimulated VSMCs, although the primary HAoSMCs and the T/G-HA-VSMC cell line do express *ASC, CASP1, CASP5,* and *Pro-IL-1β* transcripts (data will be published elsewhere). The reason for the discrepancy between AIM2 protein expression in situ and in vitro is not clear. Endogenous AIM2 is known to be expressed at very low levels and to be rather unstable (personal communication to S.D.), which might result in fast degradation in protein lysates derived from cell cultures.

Alternatively, *AIM2* expression might result in activation of other inflammatory pathways in VSMCs. IFN-γ stimulation has previously been shown to induce major histocompatibility class II transactivator (CIITA) isoforms III and IV in aortic VSMCs, resulting in activation of HLA II protein.[34] As mentioned above, this is in line with our previous observation demonstrating that AIM2 can mediate upregulation of HLA class II and CIITA isoform IV.[20] Our future investigation, using $AIM2^{-/-}$ and other mouse models of enhanced vascular inflammation will help to elucidate the precise role of AIM2 in VSMCs and explore if it has a causal linkage with atherosclerosis or other vascular pathologies that are associated with chronic inflammation.

CONCLUSIONS

The AIM2 expression pattern in macrovascular ECs and VSMCs identified here and its upregulation in response to the most prominent proinflammatory cytokines involved in atherosclerosis suggest a functional involvement of AIM2 in vascular pathogenesis. These findings provide new insight on the molecular mechanisms of vascular inflammation beyond the role of AIM2 and inflammasomes in

macrophages, and thus widen the range of putative prognostic and/or therapeutic targets in atherosclerosis.

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AUTHOR CONTRIBUTIONS

Conception and design: SD
Analysis and interpretation: SD, MH
Data collection: AB, SD, MH, AP
Writing the article: SD
Critical revision of the article: MH, AP, DB
Final approval of the article: SD
Statistical analysis: Not applicable
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Overall responsibility: SD, DB

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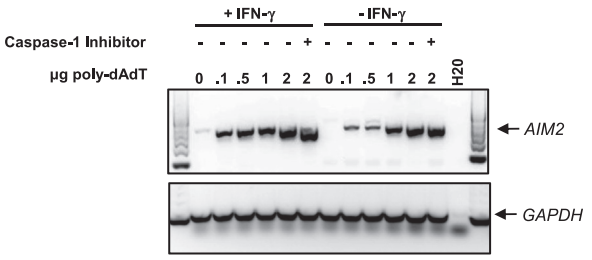
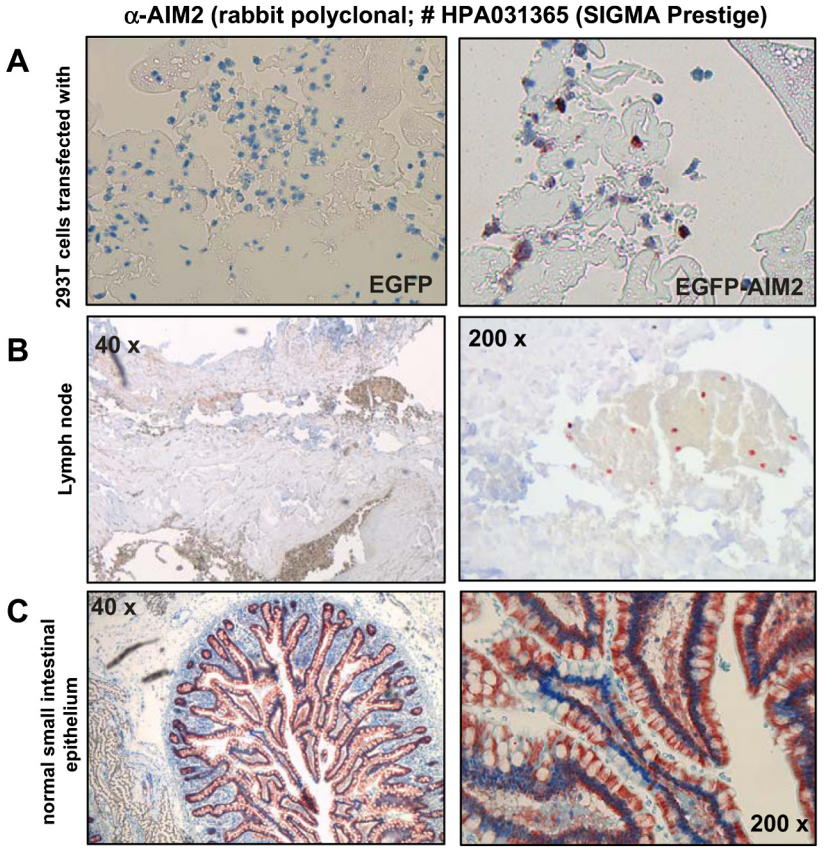


Supplementary Fig 1 (online only). Induction *absent in melanoma 2* (AIM2) transcript expression by cytoplasmic DNA in HT-29 colorectal cancer cells. Reverse transcription polymerase chain reaction was performed as described earlier.[18] *GAPDH,* Glyceraldehyde-3-phosphate dehydrogenase; *IFN,* interferon.



Supplementary Fig 2 (online only). Characterization of anti-absent in melanoma 2 (*AIM2*) antibody for immunohistochemical analysis. **A,** For characterization of AIM2 detection by the anti-AIM2 antibody, 293T cells were transfected with empty enhanced green fluorescent protein (*EGFP*) vector or a plasmid encoding EGFP-AIM2-fusion protein. Cells were formalin-fixed, precipitated in bovine serum albumin, and embedded in paraffin according to standard procedures. Immunohistochemistry was performed as described in the Methods section. Dilution of anti-AIM2: 1:150. **B,** Detection of AIM2 in single cells of a lymph node. Dilution of anti-AIM2: 1:150. **C,** Detection of AIM2 in normal small intestinal epithelium. Strong cytoplasmic expression of AIM2 is detectable in epithelial cells lining the lumen. Dilution of anti-AIM2: 1:150. Original magnification ×40 or ×200, as indicated.

Supplementary Table (online only). Sample characteristics and demographic data of patients and donors

Sample ID	*Diagnosis*	*Age (years)*	*Sex*	*Diabetic*	*Nicotine abuse*	*AIM2 around necrotic core*
Carotid lesion						
1	Stenosis; sy	78	M	N	Y	Y
2	Stenosis; sy	81	M	N	N	Y
3	Stenosis; sy	61	F	Y	N	Y
4	Stenosis; as	74	F	Y	N	N/A
5	Stenosis; sy	49	M	N	Y	Y
6	Stenosis; as	55	M	N	N	Y
7	Stenosis; as	75	F	Y	N	N/A
8	Stenosis; as	58	M	Y	N	Y
9	Stenosis; as	75	F	N	Y	Y
10	Stenosis; as	79	M	N	N	Y
11	Stenosis; as	64	F	N	Y	Y
12	Stenosis; sy	84	M	Y	N	Y
13	Stenosis; sy	50	F	Y	N	Y
14	Stenosis; as	77	M	Y	Y	Y
15	Stenosis; sy	64	M	Y	N	Y
16	Stenosis; as	76	M	Y	N	N/A
17	Stenosis; as	71	M	N	Y	Y
18	Stenosis; as	57	F	N	N	Y
19	Stenosis; sy	76	M	Y	Unknown	N/A
20	Stenosis; as	70	M	Y	Y	Y
Control	Normal	55	M	Unknown	Unknown	N/A
Aortic lesion						
1	AAA	78	M	N	N	N/A
2	AAA	84	M	N	N	N/A
3	AAA	78	M	N	N	N/A
4	AAA	69	M	N	N	N/A
Control	Normal	45	M	Unknown	Unknown	N/A

AAA, Abdominal aortic aneurysm; *AIM2*, absent in melanoma 2; *as*, asymptomatic; *F*, female; *M*, male; *N*, no (never); *N/A*, not applicable; *sy*, symptomatic; *Y*, yes (ever).